Frequent Inactivation of the *TP53* Gene in Esophageal Squamous Cell Carcinoma from a High-Risk Population in China

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the most common fatal cancers worldwide, and north central China has some of the highest rates in the world. Previous studies from tumors in this area of China have shown high frequencies of allelic loss on chromosome 17p13–11, which includes the region where the *TP53* gene is found. We examined 56 ESCC patients using single-strand conformation polymorphism and DNA sequencing to assess the frequency and spectrum of *TP53* mutation and the association between allelic loss at microsatellite marker TP53 and *TP53* mutations. Ninety-six % of cases were found to have at least one genetic alteration, including *TP53* mutation (77%), allelic loss within the *TP53* gene (73%), and/or loss of heterozygosity at the TP53 microsatellite marker (80%); 75% had two or more such alterations, including 59% with both a point mutation and an intragenic allelic loss ("two hits"). The majority of mutations observed were in exon 5, where the most common type of nucleotide substitution was a G:C→A:T or C:G→T:A transition, including half that occurred at CpG sites. Allelic loss was most commonly found in exon 4 but was very common in exon 5 as well. Taken together, the multiple genetic alterations of *TP53* in this population at high risk for ESCC indicate that there is a very high degree of genetic instability in these tumors, that *TP53* is a primary target for inactivation, and that this tumor suppressor gene plays a critical role in the carcinogenesis process for ESCC.

INTRODUCTION

ESCC[3] is one of the most common fatal cancers worldwide. There is great geographic variation in the occurrence of this tumor, including exceptional high-risk areas such as Shanxi province, a region in north central China with some of the highest esophageal cancer rates in the world (1–4). Although epidemiological studies indicate that tobacco and alcohol are the major risk factors for esophageal cancer in the low-risk regions of Europe and North America, the etiology in high-risk areas remains less clear. Several possibilities, including nitrosamines, nutritional deficiencies, fermented and moldy foods, and exposure to polycyclic aromatic hydrocarbons have been considered, but none have been convincingly linked to Shanxi's high rates of esophageal cancer (5). Previous studies in this high-risk region do, however, show a strong tendency toward familial aggregation with a consistent association between positive family history and increased risk (6–10), suggesting that genetic susceptibility may play a role in the etiology of esophageal cancer.

It is now well established that cancer development results from accumulated genetic alterations that disrupt the control of cell growth and terminal differentiation. The *TP53* gene is a critical regulator of cell growth, differentiation, and apoptosis through its actions in cell cycle checkpoint control (11). It is also well known that mutations in *TP53* are the most common genetic alterations in human cancer. The mutational spectrum of *TP53* varies by type of cancer, and for some cancer types, frequencies and/or spectra vary by different ethnic groups and nationalities (11, 12). Different carcinogens have also been associated with mutational fingerprints in *TP53*. For example, aflatoxin B_1 is associated with mutations at codon 249 in *TP53* in hepatocellular carcinoma (11). In Europe and North America, *TP53* mutations are strongly associated with tobacco smoking in esophageal cancer (13), whereas studies in China do not show elevated *TP53* mutation rates in smokers (14).

Since Mollstein *et al.* (15) first reported in 1990 that inactivation of *TP53* contributed to the development of ESCC, numerous studies have found *TP53* mutations in ESCC worldwide, including several areas of China (16–18). Only one study to date, however, has assessed the mutation frequency and spectrum of *TP53* in Shanxi, an area with exceptionally high ESCC incidence rates (18), and no one has yet reported on the association between *TP53* mutation and cancer risk factors in this population.

[3] The abbreviations used are: ESCC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism.

Table 1 Summary of genetic changes in the *TP53* gene and allelic loss in the TP53 marker

No.	Patient ID	Age/Sex	Exon/Codon	CpG site	Base change	Amino acid substitution	Designation	Lost wild-type allele within *TP53* in tumor (exon)	Genotype of *TP53* codon 72	Allelic loss (and amino acid lost) in exon 4 of *TP53* in tumor	LOH on TP53 marker
1	SHE066	51/M	7/245	No	GGC→GTC	Gly→Val	Missense	Y[a] (7)	Arg/Pro	Y (Arg)	Y
2	SHE083	64/M	5/136	No	CAA→CAG	Gln→Gln	Silent mutation in both germ-line and somatic DNA	Y (5)	Arg/Pro	Y (Pro)	Y
3	SHE216	43/F	5/176	No	TGC→TTC	Cys→Phe	Missense	Y (5)	Arg/Pro	Y (Pro)	Y
4	SHE235	59/F	6/127 and intron 6		45-bp deletion AGTGTG (gtggtgcctagagccgcctgag-intron 6-gtctggtttgcaactgggtc) TCTGGGA			Y (6)	Arg/Pro	Y (Pro)	Y
5	SHE480	65/M	5/133–135		8-bp deletion AAG (atgttttg) CCAA	Reading frame shift	Resulting in a stop at codon 145	Y (5)	Arg/Pro	Y (Pro)	Y
6	SHE516	58/M	5/175	Yes	CGC→CAC	Arg→His	Missense	Y (5)	Arg/Pro	Y (Arg)	Y
7	SHE340	50/M	8/273	Yes	CGT→CAT	Arg→His	Missense	Y (8)	Arg/Pro	Y (Arg)	Y
8	SHE057	50/F	5/143	No	GTG→ATG	Val→Met	Missense	Y (5)	Pro/Pro	HZ	Y
9	SHE081	58/M	5/146	No	TGG→TAG	Trp→Stop	Nonsense	Y (5)	Pro/Pro	HZ	Y
10	SHE384	53/F	5/166	No	TCA→TGA	Ser→Stop	Nonsense	Y (5)	Arg/Arg	HZ	Y
11	SHE052	57/M	Intron 6		CCT (AG→GG)-exon 7 GTT		At splice acceptor site	Y (intron 6)	Pro/Pro	HZ	Y
12	SHE118	56/F	7/251	No	ATC→AAC	Ile→Asn	Missense	Y (7)	Arg/Arg	HZ	Y
13	SHE123	56/M	5/153		1-bp insertion CCG (g) CCC	Reading frame shift	Resulting in a stop at codon 80	Y (5)	Arg/Arg	HZ	Y
14	SHE186	43/M	5/179	No	CAT→TAT	His→Tyr	Missense	Y (5)	Arg/Arg	HZ	Y
15	SHE240	51/M	5/127	No	TCC→TTC	Cys→Phe	Missense	N	Arg/Arg	HZ	Y
			8/273	Yes	CGT→CAT	Arg→His	Missense	Y (8)			
16	SHE328	50/F	4/75	No	CCT→TCT	Pro→Ser	Missense	Y (4)	Arg/Arg	HZ	Y
			4/116		1-bp deletion CAT (t) CT	Reading frame shift	Resulting in a stop at codon 122	Y (4)			
17	SHE263	50/M	4/55		22-bp insertion TTCA (gacgatattgaacaatggttca) CT	Reading frame shift	Resulting in a stop at codon 58	Y (4)	Arg/Pro	Y (Pro)	Y
18	SHE488	48/F	5/173	No	GTG→TTG	Val→leu	Missense	Y (5)	Arg/Pro	N	Y
19	SHE444	65/M	Intron 7		TCCTGAGT (AG→AT)-exon 8		Splice acceptor site	Y (intron 7)	Arg/Pro	Y (Arg)	HZ
20	SHE507	47/M	5/173	No	GTG→TTG	Val→Leu	Missense	Y (5)	Arg/Pro	Y (Pro)	NA
21	SHE495	54/M	5/185–186		6-bp deletion GA (tagcga) TGGT			Y (5)	Pro/Pro	HZ	HZ
22	SHE198	53/M	Intron 4		1-bp insertion CCTAC (g) AG-exon 5 TAC			Y (intron 4)	Arg/Pro	Y (Arg)	HZ
23	SHE096	57/M	7/248	Yes	CGG→TGG	Arg→Trp	Missense	N	Arg/Pro	Y (Arg)	Y
24	SHE252	57/F	8/289	No	AGA→TGA	Arg→Stop	Nonsense	N	Arg/Pro	Y (Arg)	Y
25	SHE322	50/F	5/135		4-bp insertion TTTTG (tttt) CCAA	Reading frame shift	Resulting in a stop at codon 149	N	Arg/Pro	Y (Pro)	Y
26	SHE080	39/F	4/106		1-bp deletion GCC (a) GC	Reading frame shift	Resulting in a stop at codon 112	Y (4)	Pro/Pro	HZ	HZ
27	SHE152	44/F	5/167	No	CAG→TAG	Gln→Stop	Nonsense	Y (5)	Pro/Pro	HZ	HZ
28	SHE265	49/F	8/282	Yes	CGG→TGG	Arg→Trp	Missense	Y (8)	Pro/Pro	HZ	HZ
29	SHE459	43/F	7/248	Yes	CGG→CAG	Arg→Gln	Missense	Y (7)	Pro/Pro	HZ	HZ
30	SHE495	54/M	5/185–186		6-bp deletion GA (tagcga) TGGT			Y (5)	Pro/Pro	HZ	HZ
31	SHE308	58/F	5/135	No	TGC→TGG	Cys→Trp	Missense	Y (5)	Arg/Arg	HZ	HZ
32	SHE095	58/M	8/279	No	GGG→GAG	Gly→Glu	Missense	Y (8)	Arg/Arg	HZ	N

33	SHE497	60/M	5/175	Yes	CGC→CAC	Arg→His	Missense	Y (5)	Pro/Pro	HZ	N
34	SHE069	48/M	8/273	Yes	CGT→CAT	Arg→His	Missense	N	Pro/Pro	HZ	Y
35	SHE247	45/M	4/69		2-bp deletion GAG (gc) TGCT	Reading frame shift	Resulting in a stop at codon 121	N	Arg/Arg	HZ	Y
36	SHE408	65/M	8/298		1-bp deletion GA (g) CTG	Reading frame shift	Resulting in a stop at codon 344	N	Arg/Arg	HZ	Y
37	SHE510	59/M	5/182		18-bp insertion ATGTT (tggccaactggccaagac) CTGC			N	Pro/Pro	HZ	HZ
38	SHE109	59/F	4/106	No	AGC→AGG	Ser→Arg	Missense	N	Arg/Pro	N	HZ
39	SHE027	59/M	5/176	No	TGC→TTC	Cys→Phe	Missense	N	Pro/Pro	HZ	N
40	SHE261	57/M	8/269–270		3-bp insertion AGC (aga) TTT			N	Arg/Pro	N	N
41	SHE437	47/F	5/134–140		18-bp deletion ATGTT (ttgccaactggccaagac) CTGC	Reading frame shift		N	Arg/Pro	N	N
42	SHE034	63/M	5/164–165		4-bp deletion TACT (agca) GTCA	Reading frame shift	Resulting in a stop at codon 168	N	Arg/Pro	N	NA
43	SHE138	55/F	5/174		12-bp deletion GTGAG (gcgctgccccca) CCAT	Reading frame shift		N	Arg/Pro	N	N
44	SHE021	57/F	ND					N	Arg/Pro	Y (Pro)	Y
45	SHE093	55/M	ND					N	Arg/Pro	Y (Pro)	Y
46	SHE098	53/M	ND					N	Arg/Pro	Y (Pro)	Y
47	SHE200	59/F	ND					N	Arg/Pro	Y (Pro)	Y
48	SHE360	55/M	ND					N	Arg/Pro	Y (Arg)	Y
49	SHE391	55/M	ND					N	Arg/Pro	Y (Arg)	Y
50	SHE113	43/F	ND					N	Arg/Arg	HZ	Y
51	SHE150	57/M	ND					N	Pro/Pro	HZ	Y
52	SHE409	42/M	ND					N	Arg/Arg	HZ	Y
53	SHE108	47/M	ND					N	Arg/Pro	Y (Arg)	NA
54	SHE208	56/F	ND					N	Arg/Pro	Y (Pro)	N
55	SHE273	62/M	ND					N	Pro/Pro	HZ	HZ
56	SHE170	52/F	ND					N	Arg/Arg	HZ	N

[a] Y, allele loss; N, retention; HZ, homozygous; NA, data not available; ND, no mutation detected.

In previous studies on ESCC in Shanxi province, we found frequent LOH in nearly all 30 microsatellite markers evaluated on chromosome 17p13.3–p11.2 (19, 20). The highest frequencies of allelic loss could be separated into three regions on the short arm of chromosome 17p, including 17p13.2–p13.1, where the *TP53* gene is located (21). The goals of this study were to assess the frequency and spectrum of *TP53* mutations, the association between LOH at microsatellite marker TP53 and *TP53* gene mutations, and the relation between the genetic alterations of *TP53* and cancer lifestyle risk factors and clinical/pathological characteristics in this high-risk population. Given the high frequency of LOH in the *TP53* region and previous evidence for *TP53* inactivation in ESCC, we used SSCP and DNA sequencing to examine genetic alterations of *TP53* in ESCC patients.

MATERIALS AND METHODS

Patient Selection. Patients presenting in 1995 and 1996 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, who were diagnosed with ESCC and considered candidates for curative surgical resection were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the United States National Cancer Institute. A total of 56 patients with ESCC were selected who had a histological diagnosis of ESCC confirmed by pathologists at both the Shanxi Cancer Hospital and the National Cancer Institute. None of the patients had prior therapy, and Shanxi was the ancestral home for all.

After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors, including tobacco use, frequency of alcohol, pickled vegetable and scalding hot food consumption, and a detailed family history of cancer (including all cancers in first-, second-, and third-degree relatives). Data were also recorded concerning the clinical/pathological characteristics of the patients' tumors, including location (upper, middle, lower third), pathological grade (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated), pathological Tumor-Node-Metastasis stage (I–IV), and lymph node metastasis (yes or no).

A total of 56 ESCC patients, including 34 males and 22 females, were evaluated (Table 1). Details on these ESCC patients have been described previously (21). Briefly, the mean age of the study group was 54 years (range, 39–65); 52, 57, 88, and 75% of patients reported smoking, drinking, pickled vegetable consumption, and consumption of calorically hot food, respectively. Seventy-seven % of the tumors were located in the middle third; 39% of patients had lymph node metastases; 80% of tumors were diagnosed as grade 2; and 96% of patients were Tumor-Node-Metastasis stage III at diagnosis. Thirty-four of the 56 patients had a family history of upper gastrointestinal cancer, including 24 with cancer in a first-degree relative, 8 in a second-degree relative, and 2 in a third-degree relative.

Biological Specimen Collection and Processing. Ten ml of venous blood were taken from each patient prior to surgery, and genomic DNA was extracted and purified. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser Microdissection and Extraction of DNA. Tumor cells were obtained by laser capture microdissection (Pixcell 100; Arcturus Engineering, Mountain View, CA) using methods described previously (22, 23). Briefly, unstained, ethanol-fixed, paraffin-embedded, 5-μm histological tissue sections were prepared on glass slides, deparaffinized twice with xylene, rinsed twice with 95% ethanol, stained with eosin, and air-dried. Specific cells of interest were selected from the eosin-stained slides and microdissected by laser capture microdissection. Procured cells were immediately resuspended in a 50-μl solution containing 0.01 M Tris-HCl, 1 mM EDTA, 1% Tween 20, and 0.4 mg/ml proteinase K (pH 8.0) and incubated two nights at 37°C. The mixture was then boiled for 5 min to inactive the proteinase K. Two μl of this solution were used for each PCR reaction.

PCR. DNA extracted from tumor cells microdissected from the resection specimen, and genomic DNA extracted from venous blood was used for each patient. PCR primer sets for the *TP53* gene were designed according to the nucleotide sequences obtained from the GenBank database. Nucleotide sequences of the primer sets were as follows: 5'-TCC TCT GAC TGC TCT TTT C-3' and 5'-GAA GGG ACA GAA GAT GAC AG-3' for exon 4A; 5'-CTC CTG GCC CCT GTT ATC TT-3' and 5'-CAG GCA TTG AAG TCT CAT GG-3' for exon 4B; 5'-GCC CTG ACT TTC AAC TCT GT-3' and 5'-CAG TGA GGA ATC AGA GGC-3' for exon 5; 5'-TGG TTG CCC AGG GTC CCC AG-3' and 5'-GGA GGG CCA CTG ACA ACC A-3' for exon 6; 5'-AGG CGC ACT GGC CTC ATC TT-3' and 5'-AGG GGT CAG CGG CAA GCA GA-3' for exon 7; 5'-TTG GGA GTA GAT GGA GCC T-3' and 5'-AGG CAT AAC TGC ACC CTT GG-3' for exon 8; and 5'-GCA GTT ATG CCT CAG ATT CA-3' and 5'-GGC ATT TTG AGT GTT AGA CT-3' for exon 9. The PCR primers amplified the splicing donor and acceptor sites for all exons examined. PCR reactions were carried out using a 10-μl final volume containing 1.0 μl of 10× PCR buffer I [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM MgCl$_2$], 1.0 μl of 2.5 mM deoxynucleotide triphosphate, 2 μl of DNA extraction buffer, 0.2 μl of each primer, 0.09 μl of AmpliTaq DNA polymerase (Perkin-Elmer), and 1 μCi of [α-^{32}P]dCT. Typical PCR conditions were as follows: 10 min of denaturation at 94°C, then 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An elongation step at 72°C for 10 min was added to the final cycle for exons 4, 5, 6, 8, and 9 and at 60°C for exon 7.

SSCP Analysis. The labeled PCR products were mixed with 10 μl of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) and were denatured for 10 min at 95°C and chilled on ice until loading. A 5-μl aliquot of each sample was loaded onto a 0.5× MDE gel (AT Biochem, Malvern, PA) containing 5% glycerol and run at 6W for 14–18 h at room temperature. The gel was dried on filter papers and autographed for 1–2 days using Kodak BioMax MR films.

DNA Sequencing. *TP53* PCR products that exhibited altered mobility on SSCP gels compared with normal controls were excised from the gels and eluted in 50 μl of distilled water overnight at room temperature. A 2-μl aliquot was used for PCR amplification with the same primer pair and PCR conditions used in the original PCR reaction (except without radioactivity). The second PCR products were electrophoresed on 1.0% agar-

ose gel at 100 V for 30 min, visualized by staining with ethidium bromide, and DNA purified with the Qiaquick extraction kit (Qiagen, Inc., Valencia, CA). DNA was sequenced with the Amplicycle sequencing kit from Perkin-Elmer (Branchburg, NJ) according to the manufacturer's instructions. All mutations were confirmed by repeating the entire procedure as mentioned above. Subcloning was performed in 1 case (SHE235) using the TOPO cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Statistical Analysis. All statistical analyses were performed using Statistical Analysis Systems (SAS Corp., Cary, NC). The t test (for continuous variables) and χ^2 or Mantel-Haenszel χ^2 or Fisher's exact test (for nominal variables) were used for statistical analysis of the relationship between the genetic alterations of the *TP53* gene and lifestyle risk factors as well as clinical/pathological characteristics. All *P*s were two-sided and considered statistically significant if $P < 0.05$.

RESULTS

***TP53* Mutations and Allelic Loss in ESCC Patients.** Exons 4–9 of the *TP53* gene were analyzed by PCR-SSCP for all 56 ESCC patients. Forty-five anomalous bands from tumor DNA from 43 ESCC patients were observed on SSCP gels in exons 4–8 (Fig. 1), but none were observed in exon 9. One case showed an anomalous band in DNA from both normal constitutional DNA as well as tumor DNA.

Sequence analysis of the abnormal bands found on SSCP revealed 45 mutations in *TP53*, including 2 patients with 2 different mutations each and 1 patient with the same mutation in both germ-line and tumor DNA (Table 1). The distribution of the mutations identified by location and type is summarized in Table 2. Mutations were demonstrated throughout all of the exons examined except exon 9, with the majority of mutations found in exon 5. Single mutations were also identified in introns 4, 6, and 7. Sixty-four % of the mutations were point mutations, and most of these were missense mutations (Table 2; Fig. 2*A*). A single silent point mutation was found in both the germ-line and tumor DNA in patient SHE083. Two of the three point mutations in introns 6 and 7 were located at splice acceptor sites that may affect RNA splicing. Deletions were also common ($n = 10$ or 22%; Fig. 2*B*) and ranged from 1 to 45 bp in length. Six of the 10 deletions resulted in a truncated protein. Six of the mutations (13%) were insertions (Fig. 2*C*), and all caused frameshifts in the TP53 protein coding sequence.

Among the 29 point mutations, the most common type of nucleotide substitutions were G:C→A:T and C:G→T:A transitions (55%; Table 2). Eight of these transitions were at CpG sites. G→T transversions were also common (21%), but the remainder of the substitutions varied with each type being ≤10% of the observed total.

No especially prominent pattern was seen with regard to mutations at specific codons. Three mutations were observed at codon 273, and two mutations each were found in codons 173, 175, 176, and 248. The codon 173, 175, 248, and 273 mutations occurred at CpG sites.

Overall, wild-type allelic loss was observed in 30 subjects who also had a mutation in the *TP53* gene (54% of cases). Of these 30 allelic losses, 3 occurred in exon 4, 17 in exon 5 (Fig.

1*B*), one in exon 6, four in exon 7 (Fig. 1*C*), and five in exon 8 (Fig. 1*D*).

No significant associations between *TP53* mutational status and cancer lifestyle risk factors or clinical/pathological characteristics were seen (data not shown).

Polymorphism and Allelic Loss at Codon 72 in Exon 4 of the *TP53* Gene. Sequence analysis of exon 4 products revealed a previously identified polymorphism[4] resulting from a single nucleotide substitution at codon 72 from G (Arg, upper band) to C (Pro, lower band; Fig. 1*A*). Of the 56 cases studied, 13 (23%) were homozygous for Arg, 29 (52%) were heterozygous (Arg/Pro), and 14 (25%) were homozygous for Pro (Table 1) with resultant allele frequencies of 0.49 and 0.51 for Arg and Pro, respectively.

Among the 29 informative cases (Arg/Pro heterozygotes), 22 (76%) were found to have lost an allele in the tumor, including 10 having lost Arg alleles and 12 having lost Pro alleles. Three cases with allelic loss in exon 4 of the polymorphic site also had a *TP53* mutation in another exon (one each in exons 5, 7, and 8). However, allelic loss at the polymorphic site was not significantly associated with either the presence of a *TP53* mutation ($P = 0.10$) or LOH at the TP53 microsatellite marker ($P = 0.11$). Nor were any of the cancer lifestyle risk factors or clinical/pathological characteristics we evaluated significantly associated with allelic loss at this exon 4 polymorphic site (data not shown).

Genetic Alterations in the *TP53* Gene and LOH at the TP53 Marker. Overall, genetic alterations in these ESCC patients were extremely common. These included *TP53* mutation(s) in 43 of 56 (77%) patients, wild-type allelic loss in *TP53* in 30 of 56 (54%), allelic loss at the polymorphic site in exon 4 in 22 of 29 informative cases (76%), and allelic loss at the TP53 marker in 33 of 41 informative cases (80%; Table 1). All but 2 patients (96%) had at least one alteration and 42 of 56 (75%) had multiple alterations: 7 patients had 4 such alterations, 18 patients had 3 alterations, and 17 patients had 2 alterations.

Because the TP53 marker is much simpler to examine than performing SSCP or sequencing for the *TP53* gene, we used any mutation in exons 4–9 as our gold standard to compare with the TP53 marker as a screening test for *TP53* mutation in ESCC. The TP53 marker was positive in 33 of 41 informative cases (sensitivity, 80%), but false positives were common (of 11 cases that were mutation negative, 9 were positive on TP53; specificity, 18%). Although sensitivity was reasonably high, the TP53 marker is potentially useful as a screening test only in patients who are heterozygotes for the marker, which here eliminated just over one-fourth of cases. Also, although LOH in TP53 may be a marker of *TP53* mutation, it may be a marker of another early gene.

DISCUSSION

Ninety-six % of this group of ESCC patients were found to have at least one genetic alteration, and 75% had two or more such alterations, including *TP53* mutation, allelic loss within the *TP53* gene, and/or LOH at the TP53 microsatellite marker. Two
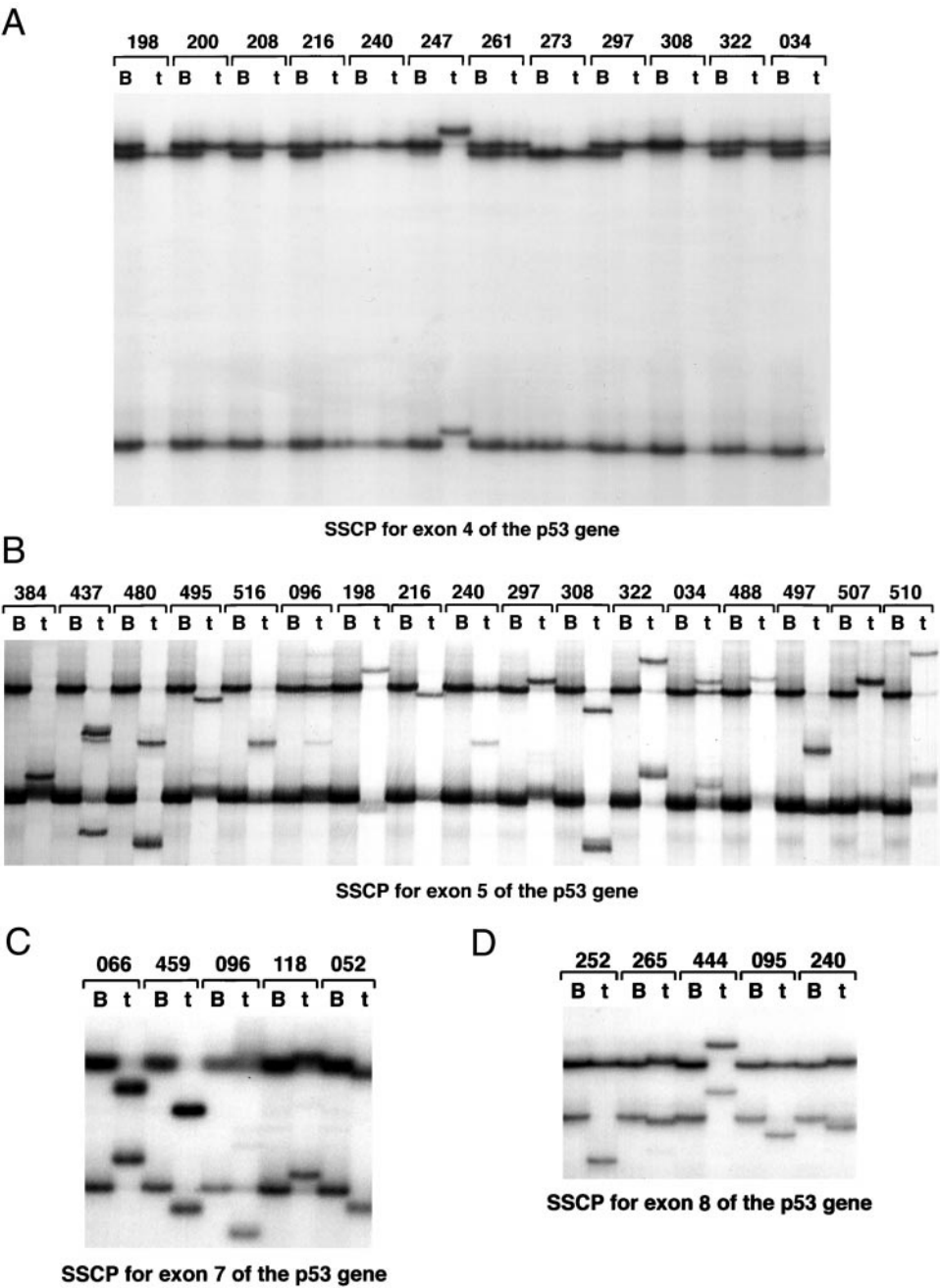
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[4] Internet address: http://www.iarc.fr/p53/poly.htm.

Fig. 1 Autoradiographs of SSCP gels demonstrating abnormal migration patterns in ESCC patients. *A*, exon 4; *B*, exon 5; *C*, exon 7; *D*, exon 8. *Lanes B*, normal DNA from blood; *Lanes t*, DNA from tumor cells.

kinds of allelic loss within the *TP53* gene were detected, wild-type allelic loss and allelic loss at a polymorphic site. To our knowledge, this is the first report to simultaneously describe *TP53* mutations, allelic loss within *TP53*, and LOH at the TP53 marker in patients with ESCC. All of the observed changes, including loss of one allele and nonsense or missense mutations in the second allele, can reduce the concentration of *TP53* tetramers. Although the changes may not cause tumors, they most likely contribute to tumor progression (24, 25). Allelic loss of *TP53* can also cause abnormally amplified centrosomes that profoundly affect mitotic fidelity and result in unequal segrega-

tion of chromosomes and enhanced genetic instability (26). Sixteen deletions and insertions were also found in this group of patients (36% of observed mutations, compared with 18–19% in other studies; Refs. 11 and 27). Deletions and insertions are more detrimental mutagenic mechanisms than single point mutations for disrupting NH_2-terminal and COOH-terminal functional domains (28). Taken together, the multiple genetic alterations of *TP53* that we detected imply that these ESCC patients had a very high degree of genetic instability and that the *TP53* tumor suppressor gene plays a critical role in ESCC carcinogenesis.

Table 2 Summary of ESCC *TP53* gene mutations in tumors by location and type

	No. of mutations	%
Location of mutations		
Exon 4	6	13
Exon 5	23	51
Exon 6	1	2
Exon 7	4	9
Exon 8	8	18
Exon 9	0	0
Introns 4, 6, 7	3	7
Total	45	100
Type of mutation		
Point mutation	29	64
Missense	24	
Nonsense	4	
Silent	1	
Deletion	10	22
Insertion	6	13
Total	45	100
Nucleotide substitutions (point mutations only)		
Transitions		
G:C→A:T/C:G→T:A	16	55
G→A	10	
C→T	6	
Transversions		
G→T	6	21
Other	7	33
C→G	3	
A→G	2	
T→A	1	
A→T	1	
Total	29	100

We detected *TP53* gene mutations in 77% of tumors in ESCC patients from this high-risk Chinese population, consistent with results from other studies of ESCC worldwide that have shown a range of *TP53* mutations from 20 to 84% (18, 29–31). The frequency of *TP53* mutations we observed was much higher than that of the only previously reported study from this same region of China (18). Lung *et al.* (18) reported *TP53* mutations in ESCC from five different geographical locals in China and found the lowest frequency (9 of 46; 20%) in Shanxi. The most common *TP53* point mutations in our study (55% of all point mutations identified) were G:C→A:T or C:G→T:A transitions. This prevalence is similar to studies of ESCC in several areas of China and India (11, 31, 32) but different from other studies in China as well as Thailand (13). No hotspot codons in *TP53* were found in our study, although two or more mutations were found in four different codons (175, 176, 248, and 273), and three of them were at CpG sites. The transitions observed were not associated with any of the risk factors examined in the present study. Differences in the results of our study compared with others who have examined *TP53* mutations in ESCC are likely attributable to several factors, including technical issues (*e.g.,* we used microdissection and examined six exons), differences in populations and/or cases studied (*e.g.,* unique or different exposures, other factors in high-risk *versus* low-risk populations, stage differences in cases studied), and the relatively small numbers of cases studied in all reports. Our small sample size is particularly relevant to our inability to find associations between mutations and risk factors.
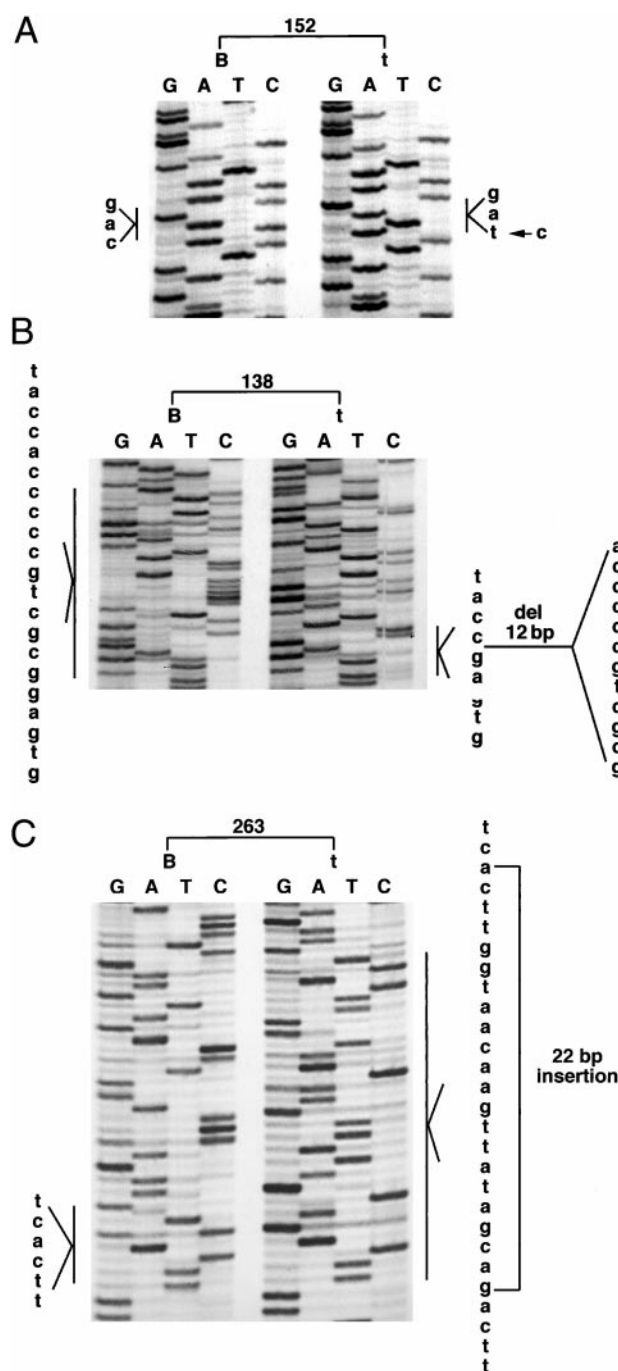

Fig. 2 Sequencing gels demonstrating *TP53* gene mutations. *A*, a nonsense mutation (CAG→TAG, at codon 167 (exon 5) resulting in Gln→stop in case SHE152. *B*, a deletion mutation (del 12 bp) at codon 174 (exon 5) resulting in reading frame shift in case SHE138. *C*, an insertion mutation (ins 22 bp) at codon 55 (exon 4) resulting in a stop code at codon 58 in case SHE263. *Lanes B,* normal DNA from blood; *Lanes t,* DNA from tumor cells.

A known *TP53* genetic polymorphism is the C→G transversion that leads to an arginine to proline change at codon 72. Several studies suggest that *TP53* polymorphisms may be associated with differences in risk for several major cancer sites,

including lung, breast, and cervix, and may also affect survival (33, 34). Other studies, however, did not reach similar conclusions (35). In the present study, codon 72 arginine and proline allele frequency was 0.49 and 0.51, respectively. These prevalences are similar to those observed in a previous study from Linxian, another ESCC high-risk area in China, where Arg/Arg, Arg/Pro, and Pro/Pro genotypes were 12, 65, and 23%, respectively (31), but are different from other populations (Arg and Pro allele frequencies are approximately 0.70 and 0.30; Refs. 33–35). Of the 29 informative patients for this polymorphism, 10 had an Arg allele loss, and 12 patients had a Pro loss. The significance, if any, of these allelic losses remains unclear.

Our results allowed us to simultaneously examine mutations and intragenic allelic loss within the *TP53* gene to determine which of our cases had two hits, in accord with the Knudsen hypothesis. We identified at least one intragenic alteration in all but 5 of the 56 cases, including 30 (54%) with both a point mutation and an intragenic wild-type allelic loss in the same exon, thus fulfilling the classic two-hit paradigm. In addition, three cases with allelic loss in the exon 4 polymorphic site also had a mutation, but in a different exon. Although the significance of these nonclassic two-hits is unknown, because we did not confirm that the allelic losses and mutations occurred on different alleles, it seems probable that both alleles were involved with consequent loss of function. Overall, a total of 33 cases (59%) had an intragenic allelic loss coupled with a mutation.

The TP53 microsatellite marker is often used as a simple screen for genetic alterations in the *TP53* gene. When we compared this marker to our SSCP and sequencing results, however, we found that the TP53 marker was reasonably sensitive in identifying cases with a mutation (80%) but was not particularly specific, testing positive in 9 cases where no mutation was identified. Possible explanations for the apparent false-positive results for the TP53 marker test include insensitivity in the SSCP test (11, 36), genetic alterations in exons or introns of the *TP53* gene that we did not examine, and that the *TP53* gene is not the sole target of the TP53 marker (37).

In summary, high frequencies of mutations and allelic loss of *TP53* were detected in 56 high-risk ESCC patients. The majority of mutations observed were in exon 5, where the most common type of nucleotide substitution was a G:C→A:T or C:G→ transition, including half that occurred at CpG sites. Allelic loss was most commonly found in exon 4. Three-quarters of these patients were found to have two or more genetic alterations. None of the cancer risk factors or clinical/pathological characteristics we evaluated was significantly associated with genetic alterations of *TP53*, although these evaluations had very low power because of our small numbers. The multiple genetic alterations of *TP53* we observed in ESCC patients from this high-risk population in Shanxi China indicate that there is a very high degree of genetic instability in these tumors and that the *TP53* gene is a primary target of LOH on 17p. Taken together, these data suggest a critical role for this tumor suppressor gene in the multistep carcinogenesis process for ESCC.

REFERENCES

1. Li, M. H., Li, P., and Li, P. R. Recent progress in research on esophageal cancer in China. Adv. Cancer Res., *33:* 173–249, 1980.

2. Li, J. Y. Epidemiology of esophageal cancer in China. Natl. Cancer Inst. Monogr., *62:* 113–120, 1982.

3. National Cancer Control Office. Investigation of Cancer Mortality in China. Beijing: People's Health Publishing House, 1980.

4. National Cancer Control Office, Nanjing Institute of Geography. Atlas of China Mortality in the People's Republic of China. Beijing: China Map Press, 1980.

5. Yang, C. S. Research on esophageal cancer in China: a review. Cancer Res., *40:* 2633–2644, 1980.

6. Li, G. H., and He, L. J. A survey of the familial aggregation of esophageal cancer in Yangcheng county, Shanxi Province. *In:* M. Wu and D. W. Neberg (eds.), Genes and Disease. Proceedings of the First Sino-American Human Genetics Workshop, pp. 43–47. Beijing: Science Press, 1986.

7. Wu, M., Hu, N., and Wang, X. Q. Familial aggregation of esophageal cancer in the high-incidence areas of North China: a strategy of control through genetic analysis. *In:* H. J. Muller and W. Weber (eds.), Familial Cancer, 1st International Research Conference, pp. 52–54. Basel: Kargen, 1985.

8. Wu, M., Hu, N., and Wang, X. Q. Genetic factor in the etiology of esophageal cancer and the strategy of its prevention in high-incidence areas of North China. *In:* H. T. Lynch and T. Hirayama (eds.), Genetic Epidemiology for Cancer, pp. 187–200. Boca Raton, FL: CRC Press, Inc., 1989.

9. Hu, N., Dawsey, S. M., Wu, M., Bonney, G. E., He, L. J., Han, X. Y., Fu, M., and Taylor, P. R. Familial aggregation of esophageal cancer in Yangcheng County, Shanxi Province, China. Int. J. Epidemiol., *21:* 877–882, 1992.

10. Hu, N., Dawsey, S. M., Wu, M., and Taylor, P. R. Family history of esophageal cancer in Shanxi Province, China. Eur. J. Cancer, *27:* 1336, 1991.

11. Greenblatt, M. S., Bennett, W. P., and Harris, C. C. Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res., *54:* 4855–4878, 1994.

12. Montesano, R. Hollstein, M., and Hainaut, P. Molecular etiopathogenesis of esophageal cancers, Vol. 32, pp. 73–84. Annals of the First Super Sanita, Lyon, France International Agency for Research on Cancer, 1996.

13. Montesano, R., and Hainaut, P. Molecular precursor lesions in oesophageal cancer. Cancer Surv., *32:* 53–68, 1998.

14. Bennet, W. P., von Brevern, M. C., Zhu, S. M., Bartsch, H., Muehlbauer, K. R., and Hollstein, M. C. p53 mutations in esophageal tumors from a high incidence area of China in relation to patient diet and smoking history. Cancer Epidemiol. Biomark. Prev., *6:* 963–966, 1997.

15. Mollstein, M. C., Metcalf, R. A., Welsh, J. A., Montesano, R., and Harris, C. C. Frequent mutation of the *p53* gene in human esophageal cancer. Proc. Natl. Acad. Sci. USA, *87:* 9958–9961, 1990.

16. Liang, Y. Y., Esteve, A., Martel-Planche, G., Takahashi, S., Lu, S. H., Montesano, R., and Hollstein, M. p53 mutations in esophageal tumors from high-incidence areas of China. Int. J. Cancer, *61:* 611–614, 1995.

17. Bennett, W. P., Hollstein, M. C., Metcalf, R. A., Welsh, J. A., Zhu, H. S., Kusters, I., Resau, J. H., Trump, B. F., Lane, D., and Harris, C. C. p53 mutation and protein accumulation during multistage human esophageal carcinogenesis. Cancer Res., *52:* 6092–6097, 1992.

18. Lung, M. L., Chan, W. C., Zong, Y. S., Tang, C. M. C., Fok, C. L., Wong, K. T., Chan, L. K., and Lau, K. W. p53 mutational spectrum of esophageal carcinomas from five different geographical in China. Cancer Epidemiol. Biomark. Prev., *5:* 277–284, 1996.

19. Hu, N., Roth, M. J., Polymeropolous, M., Tang, Z. Z., Emmert-Buck, M. R., Wang, Q. H., Goldstein, A. M., Feng, S. S., Dawsey, S. M., Ding, T., Zhuang, Z. P., Han, X. Y., Reid, T., Giffen, C., and Taylor, P. R. Identification of novel regions of allelic loss from a genome-wide scan of esophageal squamous cell carcinoma in a high-risk Chinese population. Genes Chromosomes Cancer, *27:* 217–228, 2000.

20. Hu, N., Roth, M. J., Emmert-Buck, M. R., Tang, Z. Z., Polymeropolous, M., Wang, Q. H., Goldstein, A. M., Han, X. Y., Dawsey, S. M.,

Ding, T., Giffen, C., and Taylor, P. R. Allelic loss in esophageal squamous cell carcinoma patients with and without family history of upper gastrointestinal tract cancer. Clin. Cancer Res., *5:* 3476–3482, 1999.

21. Huang, J., Hu, N., Goldstein, A. M., Emmert-Buck, M. R., Tang, Z. Z., Roth, M. R., Wang, Q. H., Dawsey, S. M., Han, X. Y., Ding, T., Li, G., Giffen, C., and Taylor, P. R. High frequency allelic loss on chromosome 17p13.3-p11.1 in esophageal squamous call carcinoma from a high incidence area in northern China. Carcinogenesis (Lond.), *11:* 2019–2026, 2000.

22. Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chuaqui, R. F., Zhuang, Z. P., Goldstein, S. R., Weiss, R. A., and Liotta, L. A. Laser capture microdissection. Science (Washington DC), *274:* 998–1001, 1996.

23. Bonner, R. F., Emmert-Buck, M. R., Cole, K. A., Pohida, T., Chuaqui, R. F., Zhuang, Z. P., Goldstein, S. R., and Liotta, L. A. Laser capture microdissection: molecular analysis of tissue. Science (Washington DC), *278:* 1481–1483, 1997.

24. Vogelstein, B., and Kinzler, K. W. p53 function and dysfunction. Cell, *70:* 523–526, 1992.

25. Udea, H., Ullrich, S. J., Gangemi, J. D., Kappel, C. A., Ngo, L., Feitelson, M. A., and Jay, G. Functional inactivation but not structural mutation of p53 causes liver cancer. Nat. Genet., *9:* 41–47, 1995.

26. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. Abnormal centrosome amplification in the absence of p53. Science (Washington DC), *271:* 1744–1747, 1996.

27. Chan, W. C., Leung, C. S., and Lilung, M. Comparative p53 mutational spectra in aerodigestive tract tumors in Hong Kong Chinese patients. Oncol. Rep., *6:* 167–172, 1999.

28. Hussain, S. P., and Harris, C. C. p53 mutation spectrum and load: the generation of hypotheses linking the exposure of endogenous or exogenous carcinogens to human cancer. Mutat. Res., *428:* 23–32, 1999.

29. Coggi, G., Bosari, S., Roncalli, M., Graziani, D., Bossi, P., Viale, G., Buffa, R., Ferrero, S., Piazza, M., Blandamura, S., Segalin, A., Bonavina, L., and Peracchia, A. p53 protein accumulation and *p53* gene mutation in esophageal carcinoma. A molecular and immunohistochemical study with clinicopathologic correlations. Cancer (Phila.), *79:* 425–432, 1997.

30. Ferec, C. I., Giroux, M. C., Audrezet, M. P., and Robaszkiewicz, M. TP53 and oesophageal cancer. Pathol. Biol. (Paris), *45:* 871–875, 1997.

31. Sti, S. T., Yang, G. Y., Wang, L. D., Xue, Z., Feng, B., Xing, E. P., and Yang, C. S. Role of *p53* gene mutations in human esophageal carcinogenesis: results from immunohistochemical and mutation analyses of carcinomas and nearby noncancerous lesions. Carcinogenesis (Lond.), *20:* 591–597, 1999.

32. Ralhan, R., Arora, S., Chattopadhyay, T. K., Shukla, N. K., and Mathur, M. Circulating p53 antibodies, *p53* gene mutational profile, and product accumulation in esophageal squamous-cell carcinoma in India. Int. J. Cancer, *85:* 791–795, 2000.

33. Bonafe, M., Olivieri, F., Mari, D., Baggio, G., Mattace, R., Ansoni, P., De Benedictis, G., De Luca, M., Bertolini, S., Barbi, C., Monti, D., and Franceschi, C. p53 variants predisposing to cancer are present in healthy centenarians. Am. J. Hum. Genet., *64:* 292–295, 1999.

34. Sun, Y., Keshava, C., Sharp, D. S., Weston, A., and McCanlies, E. C. DNA sequence variants of p53: cancer and aging. Am. J. Hum. Genet., *65:* 1779–1782, 1999.

35. Marin, M. C., Jost, C. A., Brooks, L., Irwin, M. S., O'Nions, J., Tidy, J. A., James, N., McGregor, J. M., Harwood, C. A., Yulug, I. G., Vousden, K. H., Allday, M. J., Gusterson, B., Ikawa, S., Hinds, P. W., Crook, T., and Kaelin, W. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat. Genet., *25:* 47–54, 2000.

36. White, M. B., Carvalho, M., Derse, D., O'Brien, S. J., and Dean, M. Detecting single base substitutions as heteroduplex polymorphisms. Genomics, *12:* 301–306, 1992.

37. Dunn, J., Grade, J., Dolan, K., Gosney, J. R., Sutton, R., Meltzer, S. J., and Field, J. K. Multiple target sites of allelic imbalance on chromosome 17 in Barrett's oesophageal cancer. Oncogene, *18:* 987–993, 1999.